

Minireview

A dynamic machinery for import of mitochondrial precursor proteins

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Abstract Mitochondria contain ~1000 different proteins, which are located in four different compartments, outer membrane, inner membrane, intermembrane space and matrix. The vast majority of these proteins has to be imported from the cytosol. Therefore, sophisticated molecular machineries have evolved that mediate protein translocation across or insertion into mitochondrial membranes and subsequent assembly into multi-subunit complexes. While the initial entry of virtually all mitochondrial proteins is mediated by the general import pore of the outer membrane, at least four different downstream pathways are dedicated to import and assembly of proteins into a specific compartment.

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1. Introduction

Mitochondria are ubiquitous organelles of eukaryotic cells that are surrounded by two biological membranes of distinct protein and lipid composition. They have developed during evolution from a prokaryotic endosymbiont, which was probably related to present-day α -proteobacteria. In the course of successive conversion from endosymbiont to organelle, a large fraction of the genetic information was transferred to the host's nucleus, while additional proteins encoded by the host's genome were incorporated. As a result, more than 99% of all known mitochondrial proteins are synthesized as precursors on cytosolic ribosomes and must be imported into the organelle. An additional level of complexity arises from the fact that the double-membrane bounded mitochondria consist of four different compartments, the outer membrane, the inner membrane, the intermembrane space and the matrix, which all contain a distinct set of proteins. In order to ensure efficient

and highly specific protein sorting into these compartments, an amazing variety of translocation and assembly devices using different types of energy sources are required (for an overview see Fig. 1). These complicated molecular machineries recognize specific signal sequences within preproteins and are dedicated to the biogenesis of an individual class of preproteins localized in a distinct compartment.

The initial entry gate for nearly all mitochondrial proteins is the translocase of the outer membrane (TOM complex) [1–3]. During the last years, it has become evident that at least four discrete protein sorting pathways branch off from the TOM complex. β -Barrel proteins of the outer membrane are passed on to the sorting and assembly machinery (SAM) complex [3–6], which mediates their membrane integration and incorporation into larger complexes. A considerable number of intermembrane space proteins characterized by cysteine motifs requires the mitochondrial intermembrane space import and assembly (MIA) pathway involving the recently discovered Mia40 protein and the sulfhydryl oxidase Erv1 [7–11]. Two specialized translocases of the inner membrane (TIM complexes) mediate the insertion of preproteins into the inner membrane and the translocation of preproteins into the innermost aqueous compartment, the matrix [3]. The largest class of mitochondrial preproteins contains N-terminal, cleavable signal sequences and is handed over directly from the TOM to the TIM23 complex, which is also termed presequence translocase [12–16]. These preproteins can either be sorted into the inner membrane in response to a hydrophobic stop-transfer sequence downstream of the N-terminal import signal or imported into the matrix. Inner membrane sorting depends on the proton motive force as sole energy source and involves the coupling of proton-pumping respiratory chain complexes to the TIM23 complex [17]. Translocation of large preprotein domains across the inner membrane additionally requires the association of the ATP-driven presequence translocase-associated motor (PAM) complex at the matrix side of the TIM23 complex [18–24]. The core component of the PAM complex is a mitochondrial Hsp70 (mtHsp70), which cooperates in a complicated manner with several regulatory proteins. Inner membrane proteins with multiple internal signal sequences, like metabolite carriers, are transferred from the TOM complex with the help of the TIM chaperone complex to the TIM22 complex, also named carrier translocase, which integrates these proteins into the inner membrane [3,25].

A number of recent studies have clearly demonstrated that preprotein translocases are much more than just selective, but static protein-conducting pores. We are now beginning to understand that the mitochondrial import complexes are highly

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Abbreviations: GIP, general import pore; MIA, mitochondrial intermembrane space import and assembly; PAM, presequence translocase-associated motor; SAM, sorting and assembly machinery; TIM, translocase of inner membrane; TOM, translocase of outer membrane

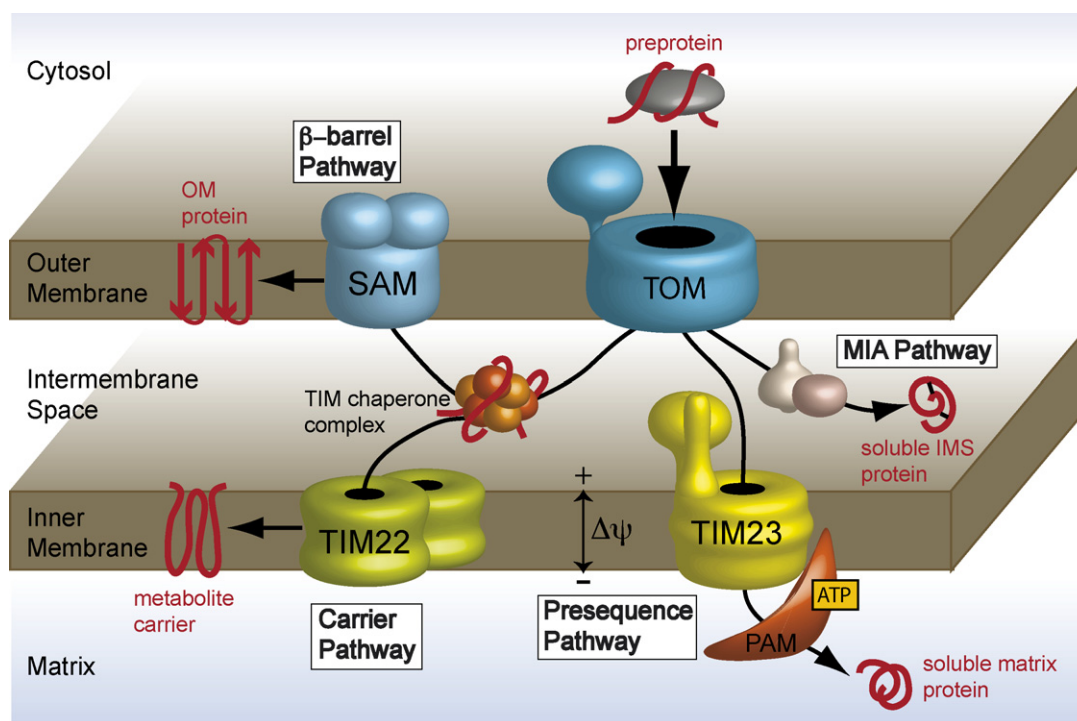


Fig. 1. The preprotein import machinery of mitochondria. Virtually all mitochondrial preproteins pass the outer membrane via the TOM complex. From the *trans* side of the TOM complex, four specific import pathways branch off, which mediate preprotein sorting into one of the four mitochondrial subcompartments: the outer membrane, the inner membrane, the intermembrane space, and the matrix. The TIM chaperone complex shields particularly hydrophobic preproteins in the aqueous environment of the intermembrane space. Assembly of outer membrane β -barrel proteins is mediated by the SAM complex (β -barrel pathway). The MIA pathway is essential for the biogenesis of numerous intermembrane space proteins. Carrier proteins of the inner membrane are transferred to the TIM22 complex with the help of the TIM chaperone complex (Carrier Pathway). Presequence-carrying proteins are imported via the TIM23 complex (Presequence pathway). Preprotein translocation into the matrix depends on the ATP-driven import motor PAM.

dynamic and versatile devices, which are tightly regulated in their activities and even subunit compositions by the import signals within preproteins. Strikingly, import components from different compartments directly cooperate to mediate transport across and into different compartments, which, like in case of presequence-carrying proteins, may involve reaction cascades and signal transduction pathways from the cytosolic surface of the mitochondria across both membranes into the matrix.

2. The TOM complex as general entry gate for mitochondrial proteins

The stable core of the TOM complex is also termed general import pore (GIP) and consists of five different proteins: the channel-forming β -barrel protein Tom40, the receptor Tom22 and the small Tom proteins Tom5, Tom6 and Tom7 [2] (Fig. 2). Two primary preprotein receptors, Tom20 and Tom70, are loosely associated with the GIP complex. While Tom70 mainly recognizes internal signal sequences within preproteins, Tom20 has a preference for preproteins with N-terminal presequences [26]. Both, Tom20 and Tom70, transfer their substrates to Tom22, which functions as a central import receptor within the GIP [1]. Tom22 delivers preproteins to the actual protein-conducting element of the TOM complex, which consists of two to three cation-selective pores with a diameter of 20–25 Å [27]. These import pores most likely consist of several molecules of Tom40. Recombinant, reconsti-

tuted Tom40 forms a single channel with similar diameter and properties as each pore of the TOM complex [28,29]. The oligomeric state of Tom40 appears to be governed by the associated subunits of the GIP. Tom5, a small protein consisting of a single transmembrane segment and a negatively charged domain exposed to the cytosol, is required for moving preproteins from Tom22 into the Tom40 channels [30]. Tom6 supports the stable interaction of Tom22 and Tom40 and appears to be generally required for maintaining the integrity of the TOM complex [31]. Tom7 was shown to affect the TOM complex in an opposite, i.e. destabilizing manner [32]. This surprising observation may be explained by the need for a certain degree of flexibility of the TOM complex in order to incorporate new subunits or it may reflect a specific step in the catalytic cycle of the TOM complex, which could be important for certain classes of substrates.

3. Biogenesis of outer membrane β -barrel proteins is mediated by the SAM complex

The mitochondrial outer membrane contains a specific class of proteins that acquire a β -barrel transmembrane topology. Such proteins are found exclusively in the outer membrane of mitochondria, chloroplasts and Gram-negative bacteria and require a specialized, evolutionary conserved machinery for their biogenesis. In yeast mitochondria, this task is fulfilled by the SAM complex [4] (Fig. 2). Interestingly, β -barrel proteins are

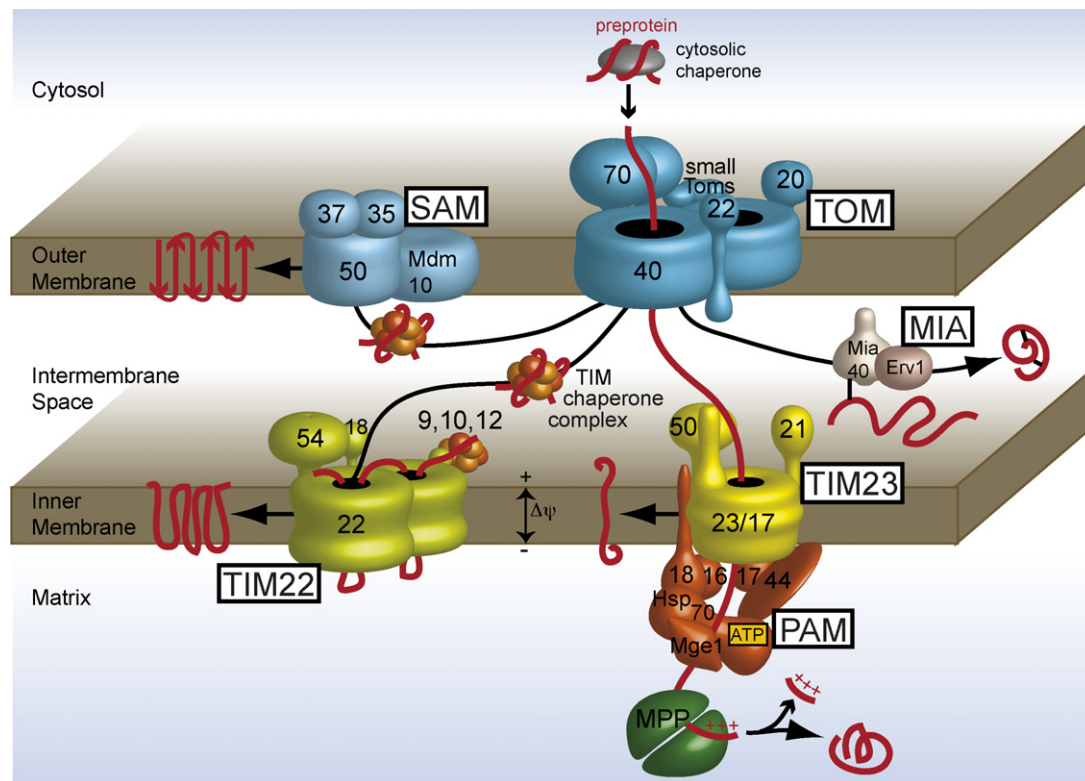


Fig. 2. Molecular architecture of mitochondrial preprotein import complexes. Cytosolic chaperones deliver mitochondrial preproteins to the import receptors of the TOM complex, Tom20 and Tom70. With the help of Tom22 they are transferred to the protein-conducting channel formed by Tom40. Mia40 and Erv1 mediate the import and oxidative folding of small intermembrane space proteins. The TIM chaperone complex of the intermembrane space has two functions: it directs β -barrel proteins to the SAM complex in the outer membrane and guides α -helical carrier proteins to the TIM22 complex in the inner membrane. The SAM complex consists of Sam35, Sam37, and the β -barrel proteins Sam50 and Mdm10. The pore-forming unit of the TIM22 complex is the Tim22 protein, which associates with Tim18, Tim54, and the adaptor protein Tim12. Presequence-carrying preproteins are directly handed over from the TOM complex to the TIM23 complex in the inner membrane. The TIM23 complex contains three essential subunits: the channel-forming Tim23 protein, Tim17, and Tim50. Preprotein-sorting into the inner membrane solely requires the TIM23 complex and the membrane potential ($\Delta\psi$). The PAM complex is essential for completion of preprotein transport into the matrix in an ATP-dependent manner. The central component of PAM is mitochondrial Hsp70, which cooperates with at least five co-chaperones: the J-protein Pam18, the J-like component Pam16, the adaptors Tim44 and Pam17, and the nucleotide exchange factor Mge1. Positively charged, matrix-targeting presequences are generally cleaved off by the matrix processing peptidase (MPP).

first translocated through the TOM complex into the intermembrane space. At the *trans* side of the TOM complex, they are bound by the TIM chaperone complex, consisting of a hexameric assembly of Tim9 and Tim10 [33]. The TIM chaperone complex guides β -barrel preproteins to the SAM complex, thereby preventing aggregation of these particularly hydrophobic polypeptides in the aqueous environment of the intermembrane space. The core component of the SAM complex is the essential β -barrel protein Sam50, also named Tob55 [5,34], which is homologous to the bacterial Omp85 protein [35]. In addition to the C-terminal β -barrel portion, Sam50 has an N-terminal hydrophilic part localized on the intermembrane space side of the outer membrane. It contains a polypeptide translocase (POTRA) domain, which was recently suggested to function as a β -barrel preprotein receptor domain and may thus take over substrates from the TIM chaperone complex [36].

Another essential subunit of the SAM complex is Sam35 (Tob38), which is a peripheral outer membrane protein exposed to the cytosol [37,38]. Temperature-sensitive mutants of *sam50* or *sam35* are defective in import and assembly of β -barrel preproteins. The third subunit of the SAM complex, Sam37 (Mas37), is not essential for life, but *sam37* deletion mutants have a β -barrel protein import defect [4]. Remarkably,

the morphology protein Mdm10 has been identified as the fourth subunit of the SAM complex [6]. While Mdm10 is required for the assembly of Tom40 into TOM complexes, it is dispensable for the biogenesis of other β -barrel proteins, like porin. Mdm10 is also found in a complex with two other known morphology proteins, Mdm12 and Mmm1 [39]. In turn, mutants in *tom40* and all SAM components show the characteristic morphological alterations of *mdm* mutants strongly suggesting an intimate link between mitochondrial protein import and morphology. It has recently been demonstrated that Tom7 functions in an antagonistic manner to Mdm10 in the biogenesis of Tom40 and porin [40]. Tom7 promotes segregation of Mdm10 from the SAM complex, while deletion of *TOM7* increases the fraction of Mdm10 found in the SAM complex. Thus, Tom7 modulates the dynamic behavior of both, the TOM and the SAM complex.

4. Many intermembrane space proteins are imported via the MIA pathway

The mitochondrial intermembrane space contains numerous small soluble proteins that possess characteristic cysteine

motifs: a twin Cx₉C motif (Cox17, Cox19, Mic14, Mic17, Mdm35), a twin Cx₃C motif (Tim8, Tim9, Tim10, Tim13), or a twin Cx₂C motif (Erv1) [7,9,11]. For Tim9 and Tim10, which form the hexameric TIM chaperone complex, it has been directly shown by X-ray crystallography that each protein contains two intramolecular disulfide bridges [41]. The import of this class of proteins into the intermembrane space requires Mia40 and the sulfhydryl oxidase Erv1 [7–11]. Upon passage through the TOM complex, substrates are initially bound to Mia40 via a transient disulfide bridge, which traps the substrates inside the intermembrane space [7] (Fig. 2). During maturation and assembly the disulfide bridge is transferred to the substrate, a process that has been named disulfide relay in analogy to the disulfide transfer machinery found in the bacterial periplasm [9,42]. The mechanistic role of Erv1, which has originally been implicated in the biogenesis of iron–sulfur cluster proteins [43], in the MIA pathway is still a matter of debate. It is clear that Erv1 is essential for the completion of the Mia40 reaction cycle. A direct, reductant-sensitive interaction of Erv1 and Mia40 has been demonstrated [9,10], and it has been suggested that Erv1 re-oxidizes Mia40 after transfer of the disulfide and substrate release [9]. However, a different study has indicated that Erv1 may act primarily on the Mia40-preprotein intermediate complex [10]. Thus, a more direct role of Erv1 in the assembly of intermembrane space proteins seems likely. Moreover, it has been proposed that cytochrome *c* is the acceptor for reducing equivalents fed into the MIA pathway [8]. Other known members of the ERV family of FAD-dependent sulfhydryl oxidases couple the oxidation of substrate sulfhydryl moieties directly with the reduction of oxygen [44].

5. Carrier proteins are integrated into the inner membrane by the TIM22 complex

Polytopic inner membrane proteins with internal targeting signals, like the large family of mitochondrial metabolite carriers, are transferred through the aqueous intermembrane space [45,46]. They are recognized in the intermembrane space by the essential Tim9–Tim10 chaperone complex. For the mitochondrial ADP/ATP carrier (AAC) it has been demonstrated that the Tim9–Tim10 complex binds to and thereby protects hydrophobic α -helical sequences within the preproteins that later become transmembrane segments [47]. Tim9–Tim10 are required for the release of carrier preproteins from the TOM complex and deliver them to the TIM22 complex. Tim22, Tim54 and Tim18 constitute the membrane-integral core of the TIM22 complex to which Tim9–Tim10 peripherally bind via the adaptor protein Tim12 (Fig. 2). This assembly represents the active TIM22 complex that can be detected as 300-kDa complex on Blue Native gels [25]. The pore-forming unit of the TIM22 complex is the Tim22 protein [25,48]. Recombinant, reconstituted Tim22 forms a voltage-activated channel that specifically responds to the addition of peptides resembling internal targeting signals [48]. The isolated TIM22 complex contains two such pores that cooperate during protein import [25]. The mechanistic roles of Tim54 and Tim18 remain to be clarified. Protein insertion via the TIM22 complex relies on the electrical potential ($\Delta\psi$) across the inner membrane as sole energy source. After an initial $\Delta\psi$ -independent tethering step, the subsequent steps occurring at the TIM22 complex are voltage-dependent, including the stable insertion of the preprotein into

one of the Tim22 pores in a loop-conformation as well as completion of membrane integration and assembly of the carrier into the mature dimeric form [25].

6. Import of presequence-carrying proteins by the TIM23 complex

The majority of mitochondrial preproteins are synthesized with positively charged, N-terminal presequences. Among them are preproteins destined for the intermembrane space, the inner membrane and the matrix and they all require the TIM23 complex for import to their final localization. The TIM23 complex consists of four membrane-embedded subunits, Tim23, Tim17, Tim50 and Tim21 [3,16] (Fig. 2). A stable complex of Tim23 and Tim17 constitutes the actual presequence protein import site at the inner membrane [12]. Both proteins show significant sequence similarity to each other and share the same topology with four transmembrane segments and N- and C-termini facing the intermembrane space. The transversal protein-conducting channel of the TIM23 complex, however, appears to consist of Tim23 alone. The electrophysiological characterization of isolated Tim23 has demonstrated that this protein forms a cation-selective, voltage-gated channel with a diameter of 13–24 Å, which specifically responds to the addition of presequence peptides [49]. In the presence of an entire presequence protein a rapid, voltage-dependent channel block is observed indicating that Tim23 alone catalyzes the initial steps of preprotein translocation. The N-terminal intermembrane space domain of Tim23 has been implicated in preprotein recognition and voltage-gating of the channel [49,50]. Based on a mutational analysis of the N-terminus of Tim17, a role of this domain in modulating the gating behavior of the Tim23 channel has been proposed [51]. This view has recently been supported by an electrophysiological study using patch clamping on mitochondrial inner membrane vesicles [52]. This work also suggested that the channel of the intact TIM23 complex is a cooperative twin-pore similar to what is observed in the TIM22 complex [25]. A careful analysis of the isolated complex will be required to clarify the physical nature of the native TIM23 complex pores and their gating properties.

How are preproteins handed over from the TOM complex to the TIM23 complex? At the *trans* side of the TOM complex, the presequence of an incoming preprotein binds to the intermembrane space domain of the central receptor Tom22 [53,54]. At this stage of import, a major part of the preprotein is still inside the Tom40 channel. The first protein of the inner membrane that contacts the incoming presequence protein is Tim50, the third essential component of the TIM23 complex [13,14] (Fig. 2). Tim50 has been crosslinked to preproteins stably arrested in the TOM complex [14] and is required for the formation of such TOM intermediates [16]. In addition to its function as a preprotein receptor at the inner membrane, Tim50 has a second role. Purified reconstituted Tim23 channels are mainly in an open state, which would cause a breakdown of the proton gradient across the inner membrane *in vivo* and thus lead to a deleterious situation in mitochondria. By a combination of *in vitro* and *in organello* studies it was demonstrated that the intermembrane space domain of Tim50 induces an oligomerization and closing of the Tim23 channel in the absence of preproteins [55]. In the presence of

presequences, the Tim23 channel is activated to allow protein translocation. Tim50 thus plays a dual role: in the absence of preproteins it is critical for maintaining the permeability barrier of the inner membrane by closing the Tim23 channel and it binds preproteins upon their translocation through the Tom40 channel and directs them to the Tim23 channel [55].

Until recently, it was assumed that the TOM and TIM23 complexes are only connected by the preprotein in transit. This view has been changed by the identification of Tim21, the first protein of the inner membrane that directly interacts with the TOM complex [16]. Purification of the TIM23 complex via an affinity tag on Tim23 revealed that Tim21 is a subunit of the presequence translocase. The protein is anchored in the inner membrane via a single transmembrane segment and exposes a large domain to the intermembrane space. In pull-down experiments the purified intermembrane space domain of Tim21 efficiently bound the TOM complex from mitochondrial detergent extracts [16,17]. The direct interaction partner of Tim21 is the intermembrane space domain of Tom22 as demonstrated by the use of a *tom22* mutant lacking this portion of the protein. Tim21 competes with presequences for

binding to Tom22 and stimulates the release of preproteins from the TOM complex, thus supporting their further transfer to the Tim23 channel in cooperation with Tim50 [16] (Fig. 3). The recently published crystal structure of the Tim21 intermembrane space domain shows a novel α/β -mixed protein fold with two α -helices flanked by an extended, eight-stranded β -sheet [56]. An important feature of Tim21 is the accumulation of positively charged conserved amino acid residues on the protein surface. These residues are likely candidates for the interaction of Tim21 with the negatively charged intermembrane space domain of Tom22.

7. Completion of matrix import depends on the ATP-driven import motor PAM

Most presequence-carrying preproteins are imported across the inner membrane into the matrix. As soon as a preprotein has been detached from Tom22 with the help of Tim21, it is inserted into the Tim23 protein-conducting channel. The only energy source required for this step is the $\Delta\psi$. Completion of

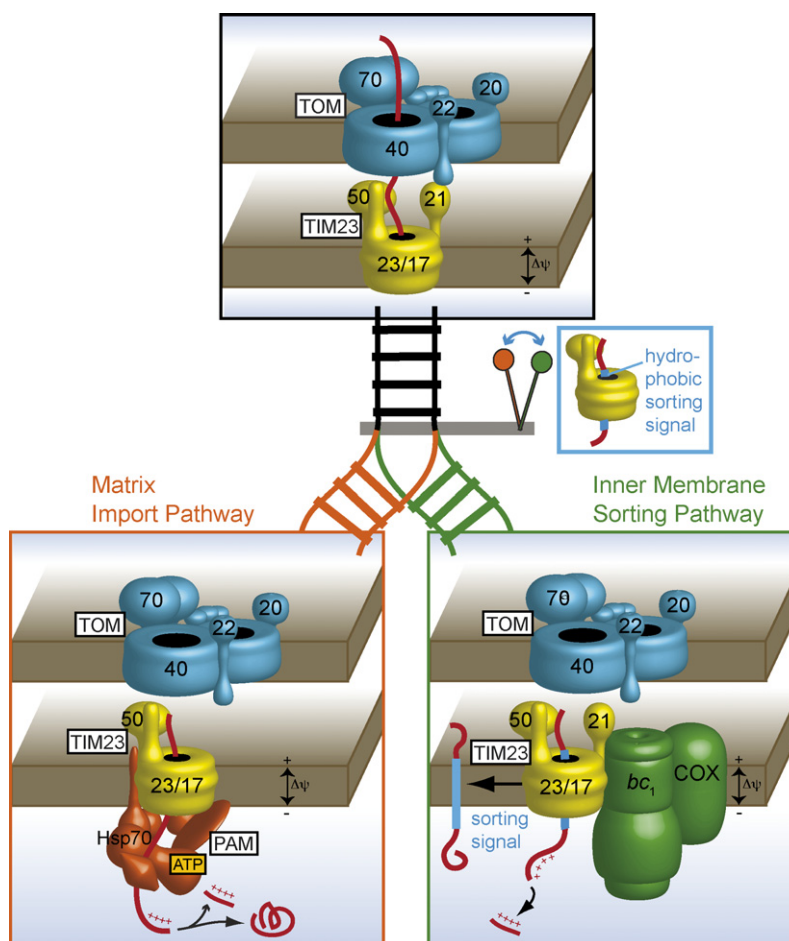


Fig. 3. The TIM23 complex switches between an inner membrane sorting form and a matrix import form (track switch model). At an early stage of preprotein import, the TOM complex and the TIM23 complex directly associate to form a supercomplex for protein transfer from the outer to the inner membrane. At the level of the inner membrane, the TIM23 complex switches between two distinct forms depending on the signal information within the incoming substrate protein. Preproteins with an additional hydrophobic sorting signal associate with a PAM-free form of the TIM23 complex that is coupled to the cytochrome bc_1 complex and cytochrome c oxidase (COX) via Tim21. This complex form catalyzes $\Delta\psi$ -dependent preprotein sorting into the inner membrane. For preprotein import into the matrix, the TIM23 complex has to switch gears. It releases Tim21 and recruits the ATP-driven import motor PAM.

preprotein import into the matrix, however, requires the association of an ATP-powered import motor, which has been named PAM complex (Fig. 2). The central component of the PAM complex is mitochondrial Hsp70 (mtHsp70), which generates an inward-directed import driving force at the expense of ATP hydrolysis [3,57,58]. As this energetically costly activity must be tightly regulated in space and time, mtHsp70 cooperates with a number of regulatory proteins. In fact, the operation of the import motor is an Hsp70 reaction cycle of unusual complexity. For a long time it has been known that mtHsp70 is associated with the TIM23 complex via the essential adaptor protein Tim44 [3,58]. The soluble matrix protein Mge1 was recognized as the nucleotide exchange factor of mtHsp70. Much later, the long-sought J-protein stimulating the ATPase activity of mtHsp70 during preprotein import has been identified as Pam18 (Tim14) [18–20]. The co-chaperone Pam18 consists of a single transmembrane span, a matrix-located J-domain and a short intermembrane space domain, which has been shown to bind to Tim17 [20]. Pam18 is tightly associated with the regulatory protein Pam16 (Tim16), which appears to be involved in recruiting Pam18 to import sites [21,22]. Pam16 is a peripheral membrane protein bound to the matrix side of the inner membrane. It contains a degenerated J-domain in which the HPD (histidine–proline–aspartate) signature motif is altered. Pam16 has been demonstrated to counteract the Hsp70 ATPase-stimulating activity of Pam18 in vitro and may thus play a crucial role in the tight regulation of the import motor [59]. Temperature-conditional mutants of both, *pam18* and *pam16*, exhibit defects in matrix import, while the PAM-independent sorting of preproteins into the inner membrane is not affected [20,21]. The recently published crystal structure of the soluble matrix portion of the Pam16–Pam18 module of the PAM complex showed an elongated complex, in which Pam16 and Pam18 are tightly packed and arranged in a back-to-back manner [24]. The interface corresponds to 20% of the total surface of each protein. A long arm protrudes from the main body of Pam18 in front of its J-domain that embraces Pam16. The phenylalanine residues flanking this arm are buried in two distinct hydrophobic pockets of Pam16. This very stable complex is not able to stimulate the ATPase activity of mtHsp70 confirming earlier data on the role of Pam16. The structure of this truncated Pam16–Pam18 module does, however, not explain how this complex can be dissociated or at least significantly rearranged, so that Pam18 becomes available for stimulation of mtHsp70 in the import reaction cycle. Therefore, the conformation of the full-length proteins including their N-terminal domains that are bound to an active TIM23 complex may be significantly different.

The situation is additionally complicated by the fact that the PAM complex contains yet another subunit, Pam17, which is required for the stable association and functional interaction of the Pam16–Pam18 module with the TIM23 complex [23] (Fig. 2). Pam17 spans the inner membrane twice with the major hydrophilic domain exposed to the matrix side. Although the protein is not essential, the deletion of *PAM17* causes specific matrix import defects similar to those observed with *pam18* and *pam16* temperature-conditional mutants. The mechanistic role of Pam17 in the PAM reaction cycle is currently unclear. The Zim17 (Hep1/Tim15) protein was also proposed to be directly involved in the import of matrix proteins [60,61]. Two subsequent studies, however, have convincingly

demonstrated that Zim17 acts indirectly on protein import by preventing mtHsp70 from aggregation [62,63].

8. Distinct forms of the TIM23 complex catalyze matrix import or inner membrane sorting

Some presequence-carrying preproteins contain a bipartite signal sequence, in which the N-terminal matrix-targeting signal is followed by a stop-transfer sequence that arrests translocation and induces a lateral sorting of the preprotein into the inner membrane. For some sorted proteins, like D-lactate dehydrogenase, the stop-transfer sequence remains part of the mature protein serving as membrane anchor. In other cases, the stop-transfer sequence of the proteins is processed by an intermembrane space peptidase that removes the membrane anchor and releases a soluble protein into the intermembrane space, like in case of cytochrome *b₂*. It is well established that preprotein sorting into the inner membrane does not require the activity of the PAM complex indicating that inner membrane sorting and matrix import are mechanistically different processes. Chacinska et al. [16] were able to demonstrate that these two processes are indeed mediated by two different forms of the TIM23 complex (Fig. 3). These authors found that Tim21 has been released from the TIM23/PAM supercomplex catalyzing matrix import. Moreover, they purified a novel isoform of the TIM23 complex that contains Tim21, but is PAM-free and showed that this complex is specifically associated with a sorted preprotein in transit. These data strongly suggest that the Tim21-containing, PAM-free form of the TIM23 complex is responsible for preprotein sorting into the inner membrane and was therefore termed TIM23^{SORT}. Thus, the TIM23 complex switches between two different forms, a sorting-competent form characterized by the presence of Tim21 and a matrix import-competent form to which the PAM complex is bound (TIM23/PAM). This switch is governed by the targeting information within incoming preproteins (Fig. 3).

The sole energy source for TIM23^{SORT} in the absence of PAM is the proton motive force across the inner membrane. Interestingly, TIM23^{SORT} was found associated with respiratory chain supercomplexes consisting of the cytochrome *bc₁* complex and cytochrome *c* oxidase (COX) [17] (Fig. 3). This interaction is at least partially mediated by the intermembrane space domain of Tim21, which was able to pull down entire respiratory chain supercomplexes from mitochondrial detergent extracts. Moreover, crosslinking experiments revealed that Tim21 is in close proximity to the cytochrome *bc₁* complex subunit 6 (Qcr6) in *organello*. This association of TIM23^{SORT} with the respiratory chain promotes the insertion of sorted preproteins into the inner membrane, which becomes particularly important when the energetic status of mitochondria is critical. Therefore, it was proposed that the respiratory chain supercomplexes are recruited to the sites of preprotein sorting by Tim21 to ensure a locally high proton-motive force, which is a prerequisite for efficient inner membrane sorting [17].

9. Open questions

After a period that has been dominated by reports on the discovery of novel pathways, components and complexes, the current main challenges in the field of mitochondrial protein

import are to understand the mechanistic roles of these new players and how they cooperate in a tightly regulated manner to achieve efficient and highly specific preprotein sorting into the different mitochondrial compartments. As outlined above, it has become evident that at least four distinct pathways branch off from the *trans* side of the TOM complex. It is unclear, how selectivity at this stage is brought about.

The mechanism of outer membrane β -barrel protein biogenesis via the SAM complex is far from understood. The molecular nature of the targeting signals within β -barrel preproteins has to be clarified. It is still an open question, at which stage of the assembly process the β -barrel structure is formed and how these proteins partition into the lipid bilayer. Why do some substrates, like Tom40, require Mdm10 for assembly, while others, like porin, do not? A role of the TIM chaperone complex in β -barrel sorting has been reported, suggesting that substrates encounter the SAM complex from the intermembrane space side of the outer membrane (Fig. 2). Interestingly, two subunits of the SAM complex, the essential protein Sam35 as well as Sam37 expose large domains to the cytosolic side of the outer membrane. It will be tempting to analyze, what the functions of these domains are. Finally, it is currently unknown, if there is any external energy source required for membrane insertion and assembly via the SAM complex.

The MIA pathway is essential for the biogenesis of a large number of intermembrane space proteins. It is unclear what the targeting signals within these preproteins are and how preprotein transfer from the TOM complex to Mia40 is achieved. Are any of the TOM complex subunits specifically required for this transfer? Another unresolved question is, at which stage of the MIA pathway Erv1 comes into play. Does it act on free, reduced Mia40 or on a Mia40-substrate complex, thus playing a more active role in the import reaction?

Carrier proteins of the inner membrane are integrated into the lipid bilayer by the TIM22 complex. While it is generally accepted now that the Tim22 protein constitutes a protein-conducting channel, little is known about the functions of the other TIM22 complex subunits, Tim18 and Tim54. It is unknown, how the TIM22 complex releases carrier proteins laterally into the membrane. Moreover, it remains to be clarified how substrates are handed over from the TIM chaperone complex to the channel. Are membrane insertion and assembly of carriers into their mature, dimeric form independent events or are they mechanistically coupled? Additionally, it would be interesting to analyze how the TIM22 complex itself is assembled.

We have just obtained initial insights into the strikingly dynamic behavior of the TIM23 complex that switches between at least two distinct forms depending on the type of substrate it has to handle. We would now like to know where the signals that trigger switches within the TIM23 complex are perceived. What is the order of events during the switch between the TIM23/PAM and the TIM23^{SORT} form? An open question is, how Pam18 is liberated from Pam16 inhibition in the active TIM23/PAM complex. Finally, it will be explored what is the function of Pam17 in the switch and in the recruitment of PAM. Other interesting questions concern the mechanism of preprotein sorting into the inner membrane: How are transmembrane proteins laterally released from TIM23^{SORT} and what is the role of Tim17 in this process? We do not have a comprehensible picture yet, how polytopic membrane proteins with N-terminal presequence, like Oxa1, are integrated into the

inner membrane. Furthermore, it will be of particular interest to examine how and when the respiratory chain complexes are coupled to TIM23^{SORT}. As binding of Tim21 to Tom22 or respiratory chain complexes appears to be mutually exclusive [17], it remains to be elucidated, which signals govern the recruitment of respiratory chain complexes to TIM23^{SORT}. Finally, it will be necessary to directly test the hypothesis of improved proton motive force utilization by the TIM23^{SORT} complex through respiratory chain coupling. As an electrical field propagates from the site of charge separation very fast, it seems to be an attractive model that high local proton gradients and possibly proton fluxes through the TIM23 complex are involved in the derivation of energy for protein import from the membrane potential across the inner membrane.

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